Exhibit E2

The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response

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It is widely believed that the hepatitis B virus (HBV) is completely cleared by antiviral antibodies and specific cytotoxic T lymphocytes (CTLs) during acute viral hepatitis. We now demonstrate that traces of HBV are often detectable in the blood for many years after clinical recovery from acute hepatitis, despite the presence of serum antibodies and HBV-specific CTLs, which can be present at acute-stage levels. The strength of the CTL response to HBV following clinical recovery correlates with persistence of HBV DNA. It is of particular interest that HBV-specific CTLs from patients studied up to 23 years after clinical and serological recovery expressed activation markers (HLA-DR, CD69) indicating recent contact with antigen. These results suggest that sterilizing immunity to HBV frequently fails to occur after recovery from acute hepatitis and that traces of virus can maintain the CTL response for decades following clinical recovery, apparently creating a negative feedback loop that keeps the virus under control, perhaps for life.

A vigorous, polyclonal and specific cytotoxic T-lymphocyte response against multiple hepatitis B virus epitopes is readily detectable in the peripheral blood during acute self-limited HBV infection[1-4]. In contrast, the CTL response is weak or undetectable in the face of the high viral load characteristic of chronic HBV infection.

Recently, we reported that traces of virus can be detected in the peripheral blood of hepatitis B surface antigen (HBsAg)-negative patients long after they have recovered from HBV infection, cleared all viral antigens and developed antiviral antibodies[4,5]. Although the basis for trace viral persistence is not understood, it is likely that immunological mechanisms control the viral burden and prevent reactivation of disease in these otherwise healthy individuals. While the antibody response to HBV probably prevents viral spread in this setting, the possibility that the CTL response to HBV might participate in this process is unexplored.

In the current study, we demonstrate that HBV-specific CTLs persist in the blood for several decades after clinical and serological recovery from acute viral hepatitis. Furthermore, we show that the CTLs can be present at acute-stage precursor frequencies long after recovery, can display an activated phenotype and can coexist with trace amounts of HBV DNA. These results indicate that continuous priming of the CTL response by persistent virus or viral antigens occurs long after clinical recovery, and they suggest that the CTLs control HBV replication in these subjects since a weak CTL response corresponds with high viral load in patients with chronic hepatitis.

The HBV-specific CTL response persists for decades

In this study, we monitored the CTL responses to five previously defined HLA-A2-restricted HBV CTL epitopes[1,4,6,7] in 17 individuals who serologically and clinically recovered from acute viral hepatitis up to 30 years earlier (Table 1) and in 12 uninfected normal controls. Because detection of these responses required prior *in vitro* stimulation, the data reflect the presence of CTL precursors in the peripheral blood of these patients, not CTL effectors.

The sum of the percent cytotoxicity of eight replicate microwell cultures stimulated with the same peptide was designated the CTL response index for the peptide (CRI-P) and is shown in bold in Table 1 if it was more than 3 standard deviations (s.d.) above the mean CRI-P values in the 12 normal controls. The mean control CRI-P values for each peptide plus or minus the standard deviation, and the CRI-P cutoffs above which CTL responses in the patients were considered positive were for peptide 1: 16.2 ± 17.1, cutoff 68; peptide 2: 13.2 ± 14.8, cutoff 58; peptide 3: 32.5 ± 21.9, cutoff 98; peptide 4: 19.8 ± 16.5, cutoff 69; and peptide 5: 8.7 ± 7.7, cutoff 32. The sum of all the positive CRI-P values was designated the total CRI, as previously reported[6].

Using these stringent criteria, 11 of the 17 subjects responded to multiple HBV epitopes, whereas 5 of the remaining 6 subjects responded to a single epitope (Table 1). It is noteworthy that many of these CTL responses were very strong, frequently exceeding the control CRI-P values for the corresponding epitope by >5 s.d., and occasionally >20 s.d. The level and variability of CTL responsiveness in these subjects is similar to what we have previously reported during the hyperacute phase of viral hepati-

Table 1 CTL responsiveness and HBV DNA following acute viral hepatitis

Subject	HBV DNA (PCR) Serum	HBV DNA (PCR) PBMC	Total CRI SUM	CRI-P (peptides) 1	2	3	4	5	Years after acute hepatitis
1	neg	neg	0	37	7	4	13	6	2.2
2	neg	neg	36	53	54	17	2	**36**	4
3	neg	neg	86	0	16	13	22	**86**	1.3
4	neg	neg	159	48	**83**	4	**76**	0	3.2
5	neg	neg	272	0	**97**	2	**175**	0	0.6
6	neg	neg	315	**205**	21	**110**	36	17	23
7	neg	pos	82	0	6	0	0	**82**	30
8	neg	pos	103	**103**	11	0	29	0	5.4
9	neg	pos	136	33	44	16	**136**	1	4.2
10	neg	pos	229	1	**90**	**139**	3	20	22
11	neg	pos	258	**116**	59	17	**83**	19	2
12	pos	pos	431	**138**	**63**	**194**	8	**36**	15
13	pos	neg	740	**402**	**229**	**109**	10	10	23
14	pos	pos	788	**230**	**60**	**163**	**335**	3	4.5
15	pos	pos	1004	**472**	**295**	65	**84**	**153**	18
16	pos	pos	1287	24	**218**	**296**	**404**	**369**	2.4
17	pos	pos	1392	**558**	**264**	**528**	59	42	23

All subjects were HBsAg and HBeAg negative and, except for no. 7, anti-HBc positive. All subjects were anti-HBs positive, except nos. 6, 7, 12, 13, 15. Serum ALT activity was normal at the times indicated. CRI-P values >3 s.d. above the normal control are shown in boldface type.

tis[4,6] and is not related to HLA-A2.1 subtype differences (F.V.C. *et al.*, manuscript in preparation).

CTL response strength correlates with persistence of HBV DNA

In keeping with previous reports from this laboratory[4,5], 11 of these subjects displayed HBV DNA in their serum and/or peripheral blood mononuclear cells (PBMCs), on the basis of the nested polymerase chain reaction (PCR) reaction (Table 1). These results were compared with the total CRI values for each patient to determine whether there was a direct correlation between the strength of the CTL response and the presence of HBV DNA. As shown in Table 1, the highest total CRI values were observed in the six subjects who were positive for HBV DNA in their serum, including subjects who had resolved their hepatitis 15–23 years earlier. It is noteworthy that all of these subjects responded to four of the five HBV epitopes except subject 12 who responded to three epitopes. These observations are consistent with the notion that the CTL response in these subjects may have been maintained by continued exposure to HBV (see below). The reason for selective HBV DNA positivity of PBMCs in some subjects (Table 1) is not clear at this time, but it would appear that the presence of HBV DNA in the serum represents a stronger immunogenic stimulus for induction of HBV-specific CTLs than its presence in PBMCs. In fact, the CTL responses of the five subjects whose PBMCs alone were HBV DNA positive were indistinguishable from the HBV DNA-negative group, displaying much lower total CRI values than the subjects whose serum was positive for HBV DNA, and responding to fewer HBV peptides (Table 1).

The CTL response can reflect recent activation

To determine whether the CTL responses detected long after clinical and serological recovery reflect recent or remote activation, PBMCs were depleted of DR-positive (recently activated) T cells before *in vitro* stimulation in subjects 15 and 16. Depletion of DR-positive T cells before *in vitro* stimulation abolished the peptide-specific CTL responses to peptides 1 and 3 in subject 15 (Fig. 1, *a* and *b*) and decreased the CTL response to peptide 5 in subject 16 (Fig. 1*c*). In a separate experiment, using T cells isolated by fluorescence-activated cell sorting (FACS) from a later bleed date in subject 16, we found that all of the CTL activity segregated in the DR-positive T-cell population and was depleted from the DR-negative T cells (Fig. 1*d*). These results suggest that, consistent with the HBV DNA-positive status of these subjects, their CTLs were recently activated by exposure to virus despite the fact that they had recovered completely from acute viral hepatitis 18 (subject 15) or 2.4 (subject 16) years earlier.

The CTL response to HBV can wane after clearance of HBV DNA

Two subjects were studied sequentially for approximately 2 years (Fig. 2). Both had normalized serum alanine aminotransferase and cleared all viral antigens within 3 months after acute hepatitis B, and they are both currently entirely healthy. Subject 1 was repeatedly negative for HBV DNA by nested PCR and Southern blot analysis, as early as 1 month after clinical onset, and remained negative for the duration of follow-up. In contrast, subject 16 remained HBV DNA positive at all time points monitored. The CTL response to HBV was readily detectable in the HBV DNA-negative subject for 22 months after recovery, but it waned thereafter and was undetectable at the 26-month time point (Fig. 2). In the HBV DNA-positive patient, on the other hand, the specific CTL response remained strong during the entire follow-up period. The foregoing observations were confirmed by quantification of the CTL precursor frequency for HBV Pol 455–463 (peptide 4) in both subjects. CTL precursors specific for this epitope were present at acute-stage levels for at least 22 months in the HBV DNA-positive subject 16, whereas they decreased to nearly one-tenth over the same period in subject 1 who became HBV DNA negative very early during his episode of acute hepatitis B (see legend to Fig. 2).

Persistent CTLs recognize endogenously synthesized antigen

We previously demonstrated that the CTL response in patients with acute HBV infection is HLA class I restricted and mediated by CD8-positive T cells that recognize endogenously synthesized antigen[1–4]. The same is true in four subjects studied up to 22 years after recovery (Fig. 3). Peptide-specific lines from those individuals were shown to recognize target cells that were either stably transfected with an HBV–EBO construct[4] or transiently infected with a vaccinia virus inducing endogenous synthesis of HBV proteins (Fig. 3, *a* and *b*), even at effector–target (E:T) ratios as low as 3:1 (Fig. 3*a*). As expected, the specific cytotoxicity could be blocked by antibodies against HLA class I, specifically HLA-A2.1, and by antibodies against CD8 but not CD4 (Fig. 3*c*).

Discussion

On the basis of clearance of HBV-encoded antigens from the blood and the appearance of antiviral antibodies, it is generally

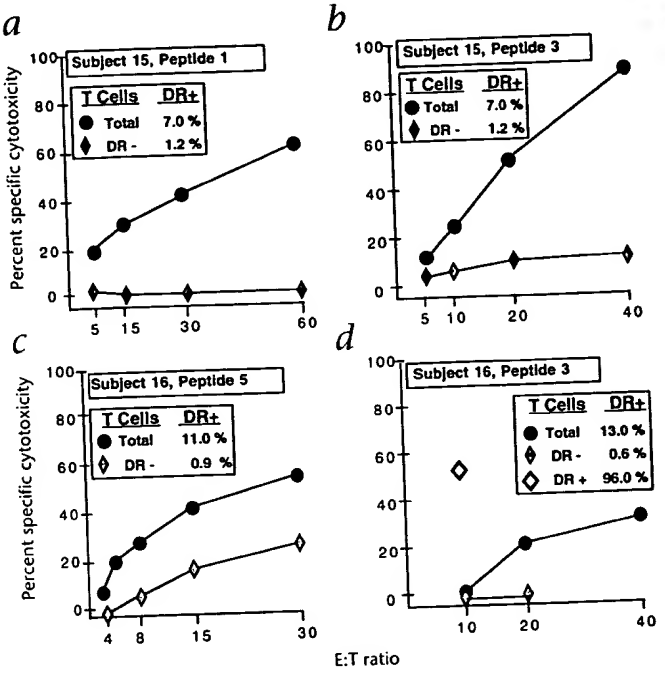

Fig. 1 Abrogation of cytolytic activity by depletion of DR-positive T cells (*a–c*) and enhancement of the cytolytic activity by enrichment for DR-positive T cells (*d*). Values for total T cells, DR-depleted T cells, and DR-enriched T cells are shown. The peptides used for stimulation are described in the text and the percentage of DR-positive T cells in each population are indicated.

assumed that HBV is completely eradicated during clinical recovery from acute hepatitis. This notion is strengthened by the disappearance of HBV DNA from the blood following recovery, using conventional solid-phase or fluid-phase hybridization assays[8,9]. Recently, however, we demonstrated that small amounts of viral DNA can be detected by nested PCR in the serum and/or PBMCs of persons convalescent from acute hepatitis for as long as 7 years after seroconversion and clinical recovery[5]. Long-term viral persistence in these individuals is not entirely unexpected, as antibodies to HBV antigens remain detectable for decades after recovery from acute viral hepatitis, often for life. Because residual viral antigens can persist in dendritic cells for very long periods[10,11]; however, persistent antibody titers need not reflect the continued presence of active virus. The induction of virus-specific CTLs, on the contrary, characteristically requires the synthesis of viral antigens by infected cells. Therefore, in the current study we asked whether the CTL response to HBV, a hallmark of acute HBV infection, persists or wanes after recovery from viral hepatitis. We also sought to determine whether there was a relationship between the strength of the CTL response and the frequency of HBV-specific CTL precursors in persons who remain HBV DNA positive by nested PCR and those who do not. Finally, we examined the CTLs for activation markers to determine whether they had recently seen antigen or reflected long-term memory of a remote infection in the distant past.

The results of this study are quite clear. CTL responses to multiple epitopes in the HBV nucleocapsid, envelope and polymerase antigens remain detectable in the peripheral blood of some individuals for two or more decades after clinical, biochemical and serological recovery from acute viral hepatitis (Table 1). It is noteworthy that in the patients whose CTLs were analyzed with activation markers, the CTL response was shown to be mediated by DR-positive T cells (Fig. 1), indicating that they had recently been activated *in vivo*, presumably by exposure to viral antigens expressed by infected cells. Finally, there is a good correlation between the persistence of viral DNA in the serum of these subjects and the overall strength and precursor frequency of the CTL response to the HBV epitopes studied (Table 1, Fig. 2), which is mediated by CD8-positive, HLA class I-restricted T cells that recognize endogenously synthesized antigen (Fig. 3).

These observations extend our earlier report[5] that HBV persists following recovery from acute viral hepatitis, and they suggest that the virus is probably transcriptionally active in these individuals because it coexists with, and actively maintains, a CTL response against its structural and nonstructural antigens. It is of interest that, in one subject who became HBV DNA negative as shown by nested PCR, the CTL response was detectable for nearly two years but waned thereafter (Fig. 2). Although these results suggest that the life span of CTLs with HBV-specific memory in this patient is on the order of 2 years and that the persistence of

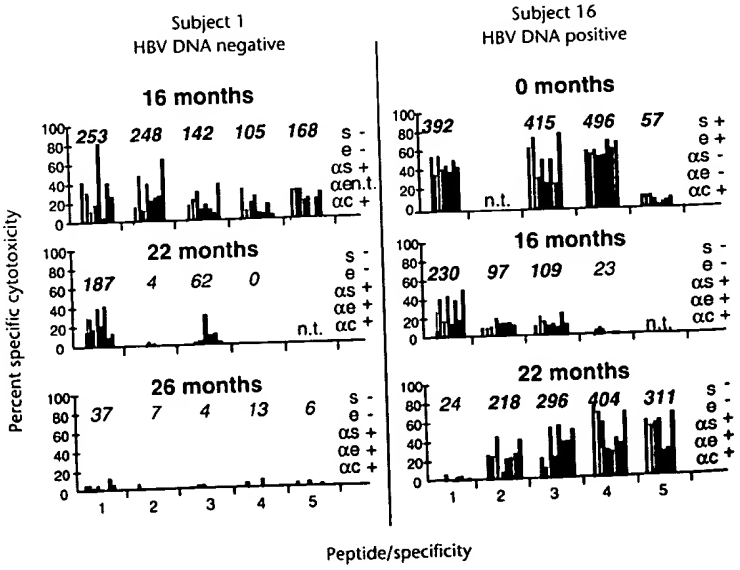

Fig. 2 HBV-specific CTL response in two subjects studied sequentially after recovery from acute hepatitis B. The peptide specificity is as described in the text, and the serological profiles at each time point are shown at the right in each panel. CRI-P values of more than 3 s.d. above the cutoff are shown in boldface type. CTL precursor frequencies were determined at selected time points. Subject 1 displayed 27 versus 3 peptide-5-specific CTL precursors per 10^6 PBMCs, respectively, 16 and 22 months after acute hepatitis B. In contrast, subject 16 displayed 18 versus 17 peptide-5-specific CTL precursors per 10^6 PBMCs, respectively, at the acute stage of disease and 22 months later. Abbreviations: s, HBsAg; e, HBV envelope antigen (HBeAg); αs, anti-HBs; αe, anti-HBe; αc, anti-HBc; n.t., not tested.

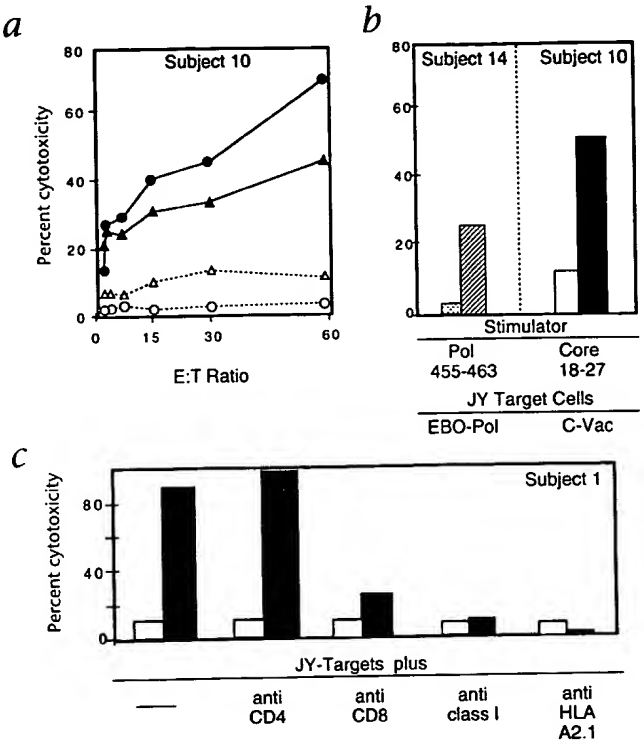

Fig. 3 *a*, HLA restriction and recognition of endogenously synthesized antigen. A CTL line stimulated with peptide 3 (Env 335–343), was tested for specific cytotoxicity against JY-EBV target cells either pulsed with 10 µg/ml of the same peptide (●—●) or without peptide (O----O), or infected with recombinant vaccinia virus vHBS4 (▲—▲) or wt-vaccinia virus (△----△). *b*, Recognition of endogenously synthesized antigen. For subject 14, an epitope-specific CTL line, stimulated with peptide 4 (Pol 455–463), was tested against a JY-EBO transfectant that stably expresses the HBV polymerase protein (hatched bar), with JY-EBO target cells as a control (stippled bar). For subject 10, a CTL line stimulated with peptide 1 (HBc 18–27) was tested for specific cytotoxicity against target cells (JY-EBV) infected with recombinant vaccinia virus (C-Vac) that expresses the HBV core protein (black bar), with wt-vaccinia virus as control (white bar). *c*, Peptide 4 (Pol 455–463) pulsed (black bar) or unpulsed (white bar) JY-EBV cells were incubated with anti-HLA-A2 (MA 2.1) or anti-class I (W6/32) monoclonal antibodies (ATCC) before the addition of peptide 4-specific CTL lines. Alternatively, anti-CD4 or anti-CD8 monoclonal antibodies were added to peptide 4-specific CTL lines before addition of the target cells as described in the text. Cytotoxicity was measured at an E:T ratio of 60:1 in a 4-h ^{51}Cr release assay.

CTLs for much longer periods of time may reflect continuous activation of the CTL response by trace amounts of virus, several other HBV DNA-negative subjects mounted CTL responses (albeit weakly) for 3.2, 4.0 and 23 years after recovery. This suggests that memory CTL responses may survive for much longer periods in the absence of antigenic stimulation in some individuals. This could not be tested directly, however, as the CTL responses in these individuals and in the subjects who displayed HBV DNA in their PBMCs but not serum were not strong enough for activation marker depletion experiments. It is relevant that the duration of a CTL response in the absence of continuous antigenic stimulation is an area of controversy at the moment[12–17].

The results of this study reinforce the concept that HBV can persist for many years after recovery from acute hepatitis. Because we only sampled the blood of these patients and because the nested PCR and CTL assays have a finite detectability threshold, it is conceivable that HBV may persist in everyone who recovers from initial infection. Our current study suggests that this infection is held in check by the CTLs, because five subjects (nos. 6, 7, 12, 13 and 15) lacked antibodies to HBsAg (Table 1). If this interpretation is correct, the results of this study raise several important issues including the possibility of viral reactivation during immunosuppression[18,19], the site (or sites) of persistent infection, the basis for incomplete clearance, the possibility of viral escape, and the likelihood that these individuals (or their organs) may be infectious for others. Indeed, several reports have confirmed that organ donors who are negative for HBsAg, positive for antibodies to hepatitis B core antigen (anti-HBc), and negative for immunoglobulin M antibodies to HBV core (HBcIgM) can transmit HBV infection[20–23].

Perhaps most important, this study illustrates that acute and chronic hepatitis during HBV infection probably represent the extremes of a biological continuum rather than the discrete entities they have previously been thought to be, and that gradations in the strength of the CTL response might influence where each patient falls in the spectrum of outcomes characteristic of HBV infection. Finally, the data suggest that active maintenance of the CTL response following acute hepatitis probably controls HBV replication in these otherwise healthy individuals, because the absence of this response correlates with high viral load in patients with chronic hepatitis.

Methods

Patient population. Seventeen HLA-A2-positive subjects with typical clinical histories and serological evidence of acute icteric viral hepatitis 0.6–30 years earlier were enrolled in this study (Table 1). HBV serology consistent with acute HBV infection was documented for all individuals except subject 7 whose laboratory results were not available for review (not shown). The serology at the time of the current analysis is described in Table 1. Results were compared with 12 HLA-A2-positive, HBV DNA-negative healthy, uninfected controls with no clinical history of HBV infection. All individuals enrolled in this study were repeatedly negative for antibodies to HCV and HIV.

Synthetic peptides, HBV antigens and expression vectors. Five peptides representing previously identified HLA-A2-restricted CTL epitopes [HBV core 18–27, FLPSDFFPSV; HBV Env 183–191, FLLTRILTI; HBV Env 335–343, WLSLLVPFV; HBV Pol 455–463 GLSRYVARL; HBV Pol 575–583 FLLSLGIHL (ref. 6)] were used in this study. Recombinant (r)HBcAg was obtained from bacterial extracts of *Escherichia coli*[24]. Recombinant vaccinia virus constructs encoding the large envelope protein (vHBS4, *adw* subtype)[6], the HBV polymerase protein (vpol, *ayw* subtype)[4,25] or the HBV core protein (C-vac, *ayw* subtype)[26] were used for transient expression of HBV proteins in human EBV-B cell lines (B-LCL) as previously described[4], and wild-type vaccinia virus[26] was used as a control.

Stimulation of PBMCs with synthetic peptides. Peripheral blood mononuclear cells were separated on Ficoll-Histopaque gradients, stimulated with synthetic peptides plus rHBcAg and recombinant human IL-2 (rIL-2) (Hoffmann–La Roche, Nutley, New Jersey) and tested for cytolytic activity on day 14 as described[6]. The remaining cells in each set of eight replicate wells were combined, restimulated and tested on days 21–24 to further characterize the CTL response.

Cytotoxicity assay and estimation of the relative strength of the CTL response. Target cells consisted of the following: JY-EBV B cells in-

cubated overnight with medium or synthetic peptides at 10 μg/ml; stable JY-EBV transfectants that express the HBV polymerase protein[27]; or JY-EBV cells infected with recombinant vaccinia viruses that express HBV proteins[4]. Cytolytic activity was determined in a standard 4-h split-well ^{51}Cr release assay, and percent cytotoxicity was determined as described[4]. Spontaneous release was less than 20% of maximum release. In selected experiments 100 μl hybridoma supernatant containing anti-HLA-A2 (MA 2.1, IgG1) monoclonal antibody or 10 μg/ml anti-class I (W6/32, IgG2α) monoclonal antibody [American Type Culture Collection (ATCC), Rockville, Maryland] were added to the target cells; alternatively, anti-CD4 or anti-CD8 monoclonal antibodies (10 μg/ml) were added to the effector cells for 30 min before starting the assay.

Limiting dilution analysis of CTL precursor frequency. For quantitative analysis of epitope-specific CTL precursors, varying numbers of PBMCs plus 1×10^5 irradiated (3000 rad) autologous PBMCs from selected donors were plated in 24 wells of a 96-well U-bottom plate containing 10 μg/ml peptide and 1 μg/ml rHBcAg, fed with 10 U/l rIL-2 on days 4, 7 and 11 and restimulated with autologous, irradiated PBMCs and peptide on day 7. A split-well CTL assay was performed on day 14. Cultures were considered positive if the specific cytotoxicity was more than 2 s.d. above the mean of the nonspecific cytotoxicity. CTL precursor frequency was calculated using the maximum likelihood method as described[28].

T-cell subset fractionation. Several experiments were performed to assess the HBV-specific CTL activity in PBMCs subsets displaying different activation phenotypes. In one experiment, T cells obtained by rosetting with AET-treated sheep red blood cells (Sigma) as previously described[29] and positively selected for CD8-positivity with magnetic beads (Dynal, Lake Success, New York) were incubated with 2.5 μg anti-DR-specific monoclonal antibody (Becton Dickinson, San Jose, California) for 60 min on ice. After washing, cells were incubated with washed GAM-Dynabeads (Dynal) at a bead–cell ratio of 50:1. DR-negative, CD8-positive T cells were recovered using a magnet and stimulated with peptide as described above. In a second experiment, purified T cells were depleted of CD4-positive and CD45RA-positive cells using magnetic beads (Becton Dickinson) according to the manufacturer's instruction. The negatively selected cells were stained with anti-DR antibody (Becton Dickinson) and sorted into DR-positive and DR-negative subpopulations on a Becton Dickinson Consort 30 flow cytometer. Efficiency of depletion was checked by FACS analysis using unlabeled anti-DR antibodies and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). DR-depleted and DR-enriched T cells were stimulated as described above. On day 21, all replicate cultures were pooled and tested for cytolytic activity over a broad E:T range. Results were compared with unfractionated T cells.

HBV DNA determination by PCR. HBV DNA was extracted from 100 μl serum and 1×10^7 PBMCs and analyzed by nested PCR using highly conserved diagnostic primer pairs exactly as described[6]. The sensitivity level was 10 HBV genome equivalents per reaction using plasmid pFC80 (ref. 30), which contains four copies of HBV DNA as a standard. Positive results were confirmed by Southern hybridization analysis using a full-length HBV DNA probe[5].

Acknowledgments

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